Bias-corrected targeted next-generation sequencing for rapid, multiplexed detection of actionable alterations in cell-free DNA from advanced lung cancer patients

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Statement of Translational Relevance

Noninvasive genotyping of cell-free plasma DNA (cfDNA) is a potentially powerful tool for advancing cancer care and translational research, but most established assays are PCR-based and limited to detection of hotspot mutations. Here, we describe the development of a novel rapid targeted next-generation sequencing (NGS) assay for study of cfDNA. Studying 48 cases using a desktop sequencer, this assay was able to detect targetable oncogenic genomic alterations and resistance mechanisms in advanced non-small cell lung cancer without any false positives. The comprehensive coverage afforded by this assay while utilizing a widely available NGS platform has great potential for broad uptake as a tool for noninvasive tumor genotyping.

Abstract

Purpose: Tumor genotyping is a powerful tool for guiding non-small cell lung cancer (NSCLC) care, however comprehensive tumor genotyping can be logistically cumbersome. To facilitate genotyping, we developed a next-generation sequencing (NGS) assay using a desktop sequencer to detect actionable mutations and rearrangements in cell-free plasma DNA (cfDNA).

Experimental Design: An NGS panel was developed targeting 11 driver oncogenes found in NSCLC. Targeted NGS was performed using a novel methodology that maximizes on-target reads, and minimizes artifact, and was validated on DNA dilutions derived from cell lines. Plasma NGS was then blindly performed on 48 patients with advanced, progressive NSCLC and a known tumor genotype, and explored in two patients with incomplete tumor genotyping.

Results: NGS could identify mutations present in DNA dilutions at ≥0.4% allelic frequency with 100% sensitivity/specificity. Plasma NGS detected a broad range of driver and resistance mutations, including *ALK, ROS1,* and *RET* rearrangements, *HER2* insertions, and *MET* amplification, with 100% specificity. Sensitivity was 77% across 62 known driver and resistance mutations from the 48 cases; in 29 cases with common *EGFR* and *KRAS* mutations, sensitivity was similar to droplet digital PCR. In two cases with incomplete tumor genotyping, plasma NGS rapidly identified a novel *EGFR* exon 19 deletion and a missed case of *MET* amplification.

Conclusion: Blinded to tumor genotype, this plasma NGS approach detected a broad range of targetable genomic alterations in NSCLC with no false positives including complex mutations like rearrangements and unexpected resistance mutations such as *EGFR* C797S. Through use of widely available vacutainers and a desktop sequencing platform, this assay has the potential to be implemented broadly for patient care and translational research.

Introduction

Genotype-directed targeted therapies are revolutionizing cancer care. Genomic alterations in genes such as *EGFR*, *ALK*, *KRAS*, and *BRAF* have been validated as powerful predictive biomarkers in the management of non-small cell lung cancer (NSCLC), colorectal cancer, and melanoma; it is now standard to test for these mutations to personalize treatment decisions.(1-7) Development of new genotype-directed therapies is widespread in solid tumor oncology, leading to increasing application of next-generation sequencing (NGS) panels that can test tumor biopsies for a wide range of potentially targetable mutations.(8, 9) However, routine use of NGS for tumor genotyping presents practical challenges including the availability of adequate biopsy specimens, slow turnaround time, and the need for repeat biopsies after development of drug resistance.(9) Given these challenges, it is clear that there is an unmet need for noninvasive assays that can broadly detect actionable genomic alterations.

Many groups, including our own, have investigated noninvasive tumor genotyping of cell-free plasma DNA (cfDNA) as an alternative to tissue genotyping.(10-15) Rather than studying circulating cells, these technologies study the free floating DNA contained in the plasma; in advanced cancer patients, a portion of this cfDNA may be derived from the tumor. Plasma genotyping has the potential to be less invasive and faster than tumor genotyping, while also allowing serial assessment of genotype during development of treatment resistance. We recently reported on a highly specific and rapid droplet digital PCR (ddPCR) assay for quantifying the concentration of *EGFR* and *KRAS* mutations in cfDNA of advanced NSCLC patients.(16, 17) Such PCR-based plasma assays test for mutations at a single site in a gene, but are limited by their inability to detect more complex genomic alterations such as chromosomal rearrangements and their inability to multiplex across several genes. Others have studied NGS of cfDNA using PCR amplicons or tagged DNA baits to enrich for target DNA sequences; however, many such assays are unable to detect rearrangements, while other assays rely on massive sequencing and computational processing resulting in unacceptable

costs and slow turnaround time.(11, 18) While detection of mutant cfDNA present at low concentration is possible with these approaches despite the more abundant wildtype (germline) DNA, a universal challenge with these highly sensitive genotyping assays is the risk of false positives due to PCR artifact.

In this study, we piloted a novel targeted NGS approach for the detection of driver mutations and rearrangements in cfDNA from advanced NSCLC patients. Taking cues from traditional hybrid capture approaches that isolate genomic subsets by pull down with probes to genes of interest**,** our methodology improves on key steps during library generation to reduce sequencing demands and turnaround times. First, to maximize on-target reads to ~90%, a two-step pull-down process was used that includes both a thermodynamically-controlled hybridization step and a kinetically-controlled extension step under conditions that neutralize GC bias. Then, to improve signal-to-noise ratio, tags were connected to each captured DNA fragment, so that every read is anchored to its clonal family and to its pull-down probe of origin, facilitating identification of low frequency mutant alleles and quantification of subtle changes in gene copy numbers (**Fig. 1, Supplemental Methods)**. We hypothesized that this approach would allow for accurate detection of a broad range of targetable genotypes, including insertions/deletions and rearrangements, in cfDNA from advanced NSCLC patients. Our goal was to leverage a desktop sequencing platform to enable a rapid turnaround time and facilitate widespread clinical adoption.

Methods

Plasma next-generation sequencing

Targeted NGS of cell-line DNA and plasma cfDNA was performed at Resolution Bioscience (Bellevue, WA) as described in **Supplemental Methods**. Chimeric gene fusions were detected using tiled probes that allow sequencing-based discovery of *de novo* rearrangements **(Supplemental Fig 1 and Supplemental Table 1)**.

Plasma ddPCR

For comparison to plasma NGS, plasma ddPCR was performed using an established and validated assay which has been described previously.(16) Briefly, this assay emulsifies extracted plasma cfDNA into thousands of droplets which subsequently undergo individual PCR with custom fluorescently labeled probes designed to detect *EGFR* L858R, *EGFR* exon 19 deletions, or *KRAS* G12X.(16) Individual droplets are then read in a flow cytometer and the number of positive droplets are quantified (BioRad). Each sample is analyzed in triplicate.

Cell line validation

The targeted NGS panel was validated using genomic DNA from 14 independent, genetically-annotated cell lines harboring four gene fusions, 19 point mutations and two insertions/deletions (**Supplemental Table 2**). Cell lines were combined into two separate DNA pools, each containing the genomes of 7 cell lines, and systematically blended with normal, wild-type DNA to produce admixtures at 2.5%, 1.0%, 0.4% and 0.1% dilutions. Prior to NGS, DNA pools were acoustically fragmented to an average size distribution centered around 165 bp and purified by two-sided SPRI to give fragment profiles of 150-200 bp that closely approximate cfDNA. Cell lines for the analytical validation experiment were obtained from ATCC (A549, H2228, SK-MEL-2, H1666, SW48), RIKEN (Lc-2/ad), the Broad institute (H1781, SW480, HCT116, H2347, HCC78) and the NCI (H3122). PC-9 and H1975 cells were obtained from the laboratory of Dr. Pasi Jänne. All cell lines were validated to be correct by short tandem repeat analyses (STR).

Patient population

Patients were identified during their routine lung cancer care at Dana-Farber Cancer Institute. Patients were deemed eligible if they had advanced NSCLC with a known tumor genotype, either untreated or progressive on therapy. Tumor genotyping was performed as part of routine care, either using conventional genotyping assays (PCR, FISH) or a targeted NGS panel when available.(5, 19) All patients consented to plasma collection and analysis on an IRB-approved prospective plasma

collection protocol or associated correlative science protocols. Following clinical validation, plasma NGS was explored in two patients with high suspicion of a targetable genotype missed on tumor genotyping.

Plasma collection

Plasma was collected prior to initiation of therapy for untreated or progressive advanced NSCLC. Whole blood was collected into 10 mL EDTA containing “purple-top” vacutainer tubes, centrifuged for 10 min at 1200g and the plasma supernatant was further cleared by centrifugation for 10 min at 3000g. Cleared plasma was stored in cryostat tubes at -80C until use. cfDNA was isolated using the QIAmp Circulating Nucleic Acid Kit (Qiagen) according to the manufacturer’s protocol. DNA was eluted in AVE buffer (100 uL) and stored at -80C until use.

Blinding of specimens

To ensure data integrity in these experiments, the samples were identified only by sample key. Only the clinical team (GRO, AGS, RSA) that identified patients for study had access to the tissue genotype results. The teams involved in ddPCR and NGS analysis were blinded during data acquisition (plasma isolation, cfDNA extraction, library generation, NGS and ddPCR analysis) and unblinding was done after NGS and ddPCR results had been reported to the clinical team.

Results

A probe set was developed that covers portions of eleven genes known to be targetable oncogenic drivers in NSCLC. Selected coding regions of eight genes were sequenced (*KRAS*, *EGFR*, *ALK*, *HER2*, *BRAF*, *NRAS*, *PIK3CA*, *MET*, and *MEK1*). Additionally, intronic probes were designed to detect genome level rearrangements that create chimeric gene fusions in *ALK*, *ROS1*, and *RET*. The coding regions of the tumor suppressor *TP53* were also included as a control because this gene is commonly mutated in NSCLC. Analysis of the performance of this probe set on plasma DNA showed >80% on-target percentage, which compares favorably with the less than 50% on-target percentage

seen in previously published data using standard hybridization selection on plasma DNA (**Supplementary Fig. 2**).(18)

The targeted NGS panel was initially validated with dilutions of cell line DNA. Four different dilutions of two DNA pools were sequenced, each derived from 7 cell lines harboring previously characterized mutations (**Supplemental Table 2**). Dilutions of 2.5% to 0.1% resulted in calculated allelic frequencies ranging from 4.5% to 0.01% (**Supplemental Fig. 3**). Variant calling algorithms (**Supplemental Methods**) were able to identify mutations that were present at 0.1% or greater with sensitivity and specificity of 88% and 100%. Diagnostic performance improved to 100% sensitivity and specificity for mutations present at an allelic frequency 0.4% or greater (**Supplemental Fig. 3**, **Supplemental Table 2**). This preliminary analysis of the cell lines allowed the setting of thresholds that ensured high specificity in the subsequent analysis of patient samples.

After validating the NGS platform with DNA dilutions, plasma samples from 48 patients with advanced NSCLC were studied, blinded to the tumor genotyping results (**Supplemental Table 3**). The median age of these patients was 57, 61% were female and 92% had extra-thoracic metastatic disease. Mean reads per sample was 8.9 million, with a mean coverage per base of 983 unique reads.

The sensitivity of the NGS platform was first studied in 29 of the 48 patients known by tumor genotyping to harbor *EGFR* and *KRAS* driver mutations readily assayed with ddPCR (**Fig. 2A**). Using the tumor genotyping as the gold standard, ddPCR of plasma had a sensitivity of 86%, while NGS had a sensitivity of 79%, not significantly different ($p=0.43$). Both methods were more sensitive when more cfDNA was available. The allelic fraction of the mutant allele in cfDNA, calculated as the number of mutant reads over wildtype reads, was closely correlated for plasma NGS and plasma ddPCR (Pearson Correlation = 0.93, $p<0.001$, **Fig. 2B**).

Detection of rare mutations and rearrangements in cfDNA was next studied in a blinded fashion in 20 of the 48 patients with NSCLC known to harbor a rare mutation or rearrangement on

tumor genotyping (**Fig. 2C**); these included 19 new cases plus one previously studied above, harboring both a *KRAS* mutation and a *PIK3CA* mutation. Plasma NGS was able to blindly detect 6 out of 8 cases with rearrangements as well as 4 cases with rare targetable *HER2* and *EGFR* mutations. Sensitivity in this cohort was 75% (15/20), similar to the prior analysis, (**Fig. 2C**). For each of the 6 rearrangements detected, the exact breakpoints and fusion partners could be mapped to the genome (**Fig. 3A**, **Supplementary Fig. 4**).

Specificity was studied in these 48 patients, each with tumor genotyping positive for an oncogenic driver mutation in *EGFR*, *KRAS*, *ALK*, *ROS1*, *RET*, *BRAF*, or *HER2*. These oncogenes are established as non-overlapping on tumor genotyping and are therefore ideal gold standards for assessment of false positives.(5, 7) Specificity of the plasma NGS platform was 100% for these seven driver genotypes, with a false positive rate of 0% (95% CI 0-10%).

Detection of resistance mutations was explored in 15 of the 48 patients who had plasma collected after development of acquired resistance to a tyrosine kinase inhibitor (**Table 1**). Of 12 patients with acquired resistance to erlotinib or afatinib, plasma NGS detected T790M in 8. One of the 12 patients had been refractory to erlotinib and afatinib despite harboring an *EGFR* exon 19 deletion, and tumor NGS had identified high *MET* amplification. Blinded to the tumor genotype, plasma NGS similarly detected *MET* amplification, as evidenced by a significant increase in *MET* copies compared to control (**Fig. 3B**). No resistance mutations were identified in the remaining 3 *EGFR*-mutant cases. Studying two patients who had developed acquired resistance to crizotinib, plasma NGS identified two point mutations in the *ALK* kinase domain in one case with an ALK rearrangement, and no *ROS1* resistance mutations in the other case with a ROS1 rearrangement. One patient was studied who had previously developed T790M-positive resistance to erlotinib, and subsequently developed acquired resistance to the investigational EGFR kinase inhibitor AZD9291;(20) plasma NGS identified two different DNA mutations encoding for *EGFR* C797S (**Fig. 3C**), a mutation recently described as a common mediator of acquired resistance to AZD9291.(21). Fourteen of fifteen cases had resistance

biopsies available for genotyping. Tumor and plasma results were concordant in 12 (86%), while in 2 cases plasma NGS did not detect an acquired T790M mutation detected in tumor. Altogether, sensitivity for the 62 known driver and resistance mutations from the 48 cases was 77% (48/62).

Lastly, the plasma NGS assay was explored in two advanced NSCLC patients with high clinical suspicion of possessing a targetable genomic alteration which had been missed on prior tissue genotyping. The initial patient was a 66 year-old female never-smoker who had responded durably to empiric treatment with erlotinib and developed resistance; *EGFR* genotyping of a re-biopsy using a commercial PCR assay had identified T790M without any sensitizing mutation evident. Plasma ddPCR was first performed and detected 1508 copies/ml of an exon 19 deletion. Plasma NGS then confirmed the presence of a novel double-deletion in exon 19 of *EGFR* which would have been missed by many commercial PCR assays because these often detect only common exon 19 deletion variants (**Fig. 3D**). The second patient was a 28 year-old female never-smoker who had progressed on multiple lines of therapy, for whom previous tumor genotyping (including NGS) had revealed no targetable alterations despite 4 biopsies. Plasma NGS revealed high level *MET* amplification which was subsequently confirmed by fluorescent *in situ* immunohistochemistry (MET:CEP7 ratio >5), and led to the initiation of crizotinib. For each case, turnaround time from blood draw to result reporting in this initial feasibility study was 6 business days.

Conclusion

In this blinded study, a targeted NGS of cfDNA from advanced NSCLC patients was able to accurately detect a broad range of targetable genomic alterations in NSCLC, including point mutations, insertions / deletions, and rearrangements,with no false positives. This report is the first, to our knowledge, to describe the accurate detection of *ALK*, *ROS1*, and *RET* rearrangements using a single plasma assay without prior knowledge of fusion partners. This represents a dramatic advance over PCR-based plasma genotyping assays which are limited to the detection of hotspot mutations in

coding regions.(10) This assay was also able to detect both canonical and novel resistance mechanisms, including *MET* amplification and *EGFR* C797S.(22, 23)

Importantly, this approach uses widely available equipment such as standard EDTA-containing vacutainers and a desktop sequencing platform: any accredited molecular pathology lab with a MiSeq could, with the right technical expertise and bioinformatics support, implement this assay to guide the care of advanced NSCLC patients.

While others have also studied plasma NGS of lung cancer, this is the first to describe comprehensive and blinded detection of a broad range of alterations with one clinic-ready assay. Detection of hotspot mutations using plasma NGS was described by Couraud et al using the IonTorrent platform, achieving a sensitivity of 58% and 87% specificity but without the ability to detect rearrangements or amplifications.(11) Newman *et al* recently demonstrated more comprehensive detection of lung cancer mutations in cfDNA and tumor tissue by hybrid capture using biotinylated oligonucleotide probes on a HiSeq;(18) however, this study noted an inefficient capture of fusion rearrangements. Finally, targeted sequencing of cfDNA using PCR amplicons has been successful for detection of SNVs in multiple types of cancer;(15) however, PCR amplicons are not trivial to multiplex and are inherently blind to gene rearrangements. The NGS platform described here overcomes the weaknesses of each of these prior approaches, allowing multiplexed detection of a broad range of alterations with no false positives using an efficient platform. Furthermore, turnaround time from time of blood draw can be as short as 6 days.

Intuitively, we found that the sensitivity of plasma NGS is improved in specimens with a higher quantity of cfDNA, though in some instances targetable genotypes could be detected in specimens with a relatively low number of GE sequenced indicating high DNA shed from the tumor. In this pilot study, sensitivity of plasma NGS was 77%, comparable to most plasma genotyping assays which have reported sensitivity in the range of 70%.(24), Sensitivity improves with higher DNA concentration, highlighting how important it will be to understand why the amount of total cfDNA in

plasma varies across such a wide range, and whether this variance is due to fluctuations in tumor biology or in methods of extraction. For example, we and others have described previously that plasma genotyping assays are more sensitive in lung cancer patients with extra-thoracic metastases.(13, 15, 17, 18) Even with a moderately high sensitivity, the lack of false positives with this assay results in a 100% positive predictive value, meaning that this plasma NGS assay could be used as a screening step before a biopsy sample is taken for genomic analysis: if positive, the results are reliable and can potentially obviate the need for a biopsy, and if negative, a biopsy for further testing may still have value.

Our detection of various resistance mechanisms in patients with acquired resistance to TKIs, including the detection of two different *EGFR* C797S clones in one case, highlights the potential power of plasma NGS for understanding the heterogeneity of the resistant state. In addition, the C797S alleles identified with plasma NGS were clearly in *cis* with T790M (Figure 3), a detail that would be difficult to decipher with a PCR assay and may have treatment implications. (22) It is increasingly appreciated that resistant tumors are made up of clones with diverse biologies that may respond differently to therapy.(25, 26) We recently showed that three molecular subtypes of acquired resistance to the investigational *EGFR* inhibitor AZD9291 are apparent by use of serial plasma ddPCR: while all patients started with T790M plus a sensitizing mutation, some lose T790M at resistance while some gain C797S.(21) However, this analysis required five separate PCR assays: T790M, 19 deletion, L858R, and assays for two C797S variants. Alternatively, one plasma NGS assay can detect these 5 alterations plus detecting any novel resistance mechanisms that emerge. Our data further suggest that quantification of allelic fraction is similar using plasma NGS and ddPCR, suggesting either could be used to serially monitor plasma genotype concentration.

The findings described in this report are achieved by applying a novel bias-corrected capture technology that builds upon standard sequencing approaches to maximize the efficiency of on-target versus off-target and redundant sequencing reads. By reducing PCR artifacts, this assay can

accurately detect mutation present in as few as 0.1% of sequencing reads; in contrast, some tumor NGS platforms are unable to accurately call a mutation unless detected in >10% of sequencing reads. Bias-corrected targeted NGS is an approach that can be applied to any sequencing platform to improve on-target coverage and reduce noise. Here, we apply bias-corrected targeted NGS to desktop sequencing on a MiSeq platform in order to develop a plasma assay with the potential to be rapid and clinic-ready, in contrast to more time intensive assays utilizing HiSeq platforms.(18) Yet this NGS approach could also be applied to clinical sequencing panels or discovery efforts to improve sequencing coverage and reduce artifact, particularly when studying clinical specimens from small biopsies with scant DNA..

In conclusion, we have developed and successfully piloted a plasma NGS assay that, using a novel capture and analysis technique, can detect targetable mutations and rearrangements in plasma from advanced NSCLC patients. This is the first plasma NGS assay to demonstrate blinded, multiplexed detection of such a broad range of actionable alterations with no false positive results. By using a widely available desktop sequencing platform and standard vacutainers with the potential for a rapid turnaround time, this assay has the potential for broad uptake and application. Through reducing the barriers between NSCLC patients and genotyping, we hope that plasma NGS will be able to facilitate delivery of targeted therapies and improve outcomes for patients with advanced NSCLC.

Fig1. Key differences between standard hybrid capture (left) and bias-corrected NGS (right). (A) Mono-, di- and trimeric nucleosome cfDNA fragments ranging from 130-480 basepairs are isolated. (B) In standard hybrid capture, cfDNA fragments are end-repaired and ligated with single primers. In contrast, bias-corrected NGS uses multifunctional adaptors that include sequences for single-primer amplification (red), tags for sample identification (green), and sequence identification tags (blue) that, in conjunction with the fragmentation site (blue dot) identify unique sequence clones. (C) In standard hybridization cfDNA fragments are captured with large capture probes (up to 120 bp) that span the genetic region of interest and may result in off-target fragments being isolated (e.g., daisy-chaining off-target DNA). Bias-corrected NGS uses small capture probes (~40 bp) that are designed to be adjacent to the region of interest. Primer extension of fragments copies genomic and adaptor sequences. Lastly, amplification with tailed PCR primers create sequencing ready clones. (D) While both approaches allow sequencing of gene re-arrangements, large capture probes designed to target one gene will inefficiently target fragments containing a large amount of fusion partner gene sequence, resulting in poor sensitivity. In bias-corrected NGS, gene junction and partner gene sequence is replicated during primer extension. E: In standard hybrid capture all pulled-down cfDNA (specific and non-specific) is amplified and sequenced without knowing the exact read or probe which captured the fragment. In bias-corrected NGS, READ_1 identifies the sample ID and the unique sequence identifiers, while READ_2 identifies the probe that pulled down each clone, facilitating read analysis and probe optimization.

Fig. 2. Plasma NGS compared to known tumor genotype across a range of genomic equivalents (GE) in the sequencing library. The mutant allele frequency is provided when detected by the plasma genotyping assay (green circle) but not if undetected (red circle). In patients with common *EGFR* and *KRAS* mutations (A) plasma NGS has similar sensitivity to plasma ddPCR. Quantification of allelic frequency with plasma NGS and plasma ddPCR are closely correlated (B). In patients with rare

genotypes (C), plasma NGS is able to detect a wide range of genomic alterations. In both groups of patients, the rate of detection by plasma NGS increases as the number of GE increases (A, C).

Fig.3. Bias-corrected NGS of cfDNA identifies complex genomic alterations. (A) Sequencing of the intronic region of *RET* detects reads extending into KIF5B (inset), predicting a fusion of these two genes. (B) In cfDNA from a case with known *MET* amplification (case 105), *MET* copy number is significantly increased compared to control probes ($p<0.001$), which is not seen in cases without *MET* amplification. (C) Two mutations encoding for *EGFR* C797S are detected in *cis* with *EGFR* T790M after resistance to AZD9291. (D) In a case with acquired T790M despite no apparent *EGFR* sensitizing mutation, plasma NGS detects a novel double-deletion in exon 19 of *EGFR* which would have been missed with many PCR-based genotyping assays.

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Figure 1

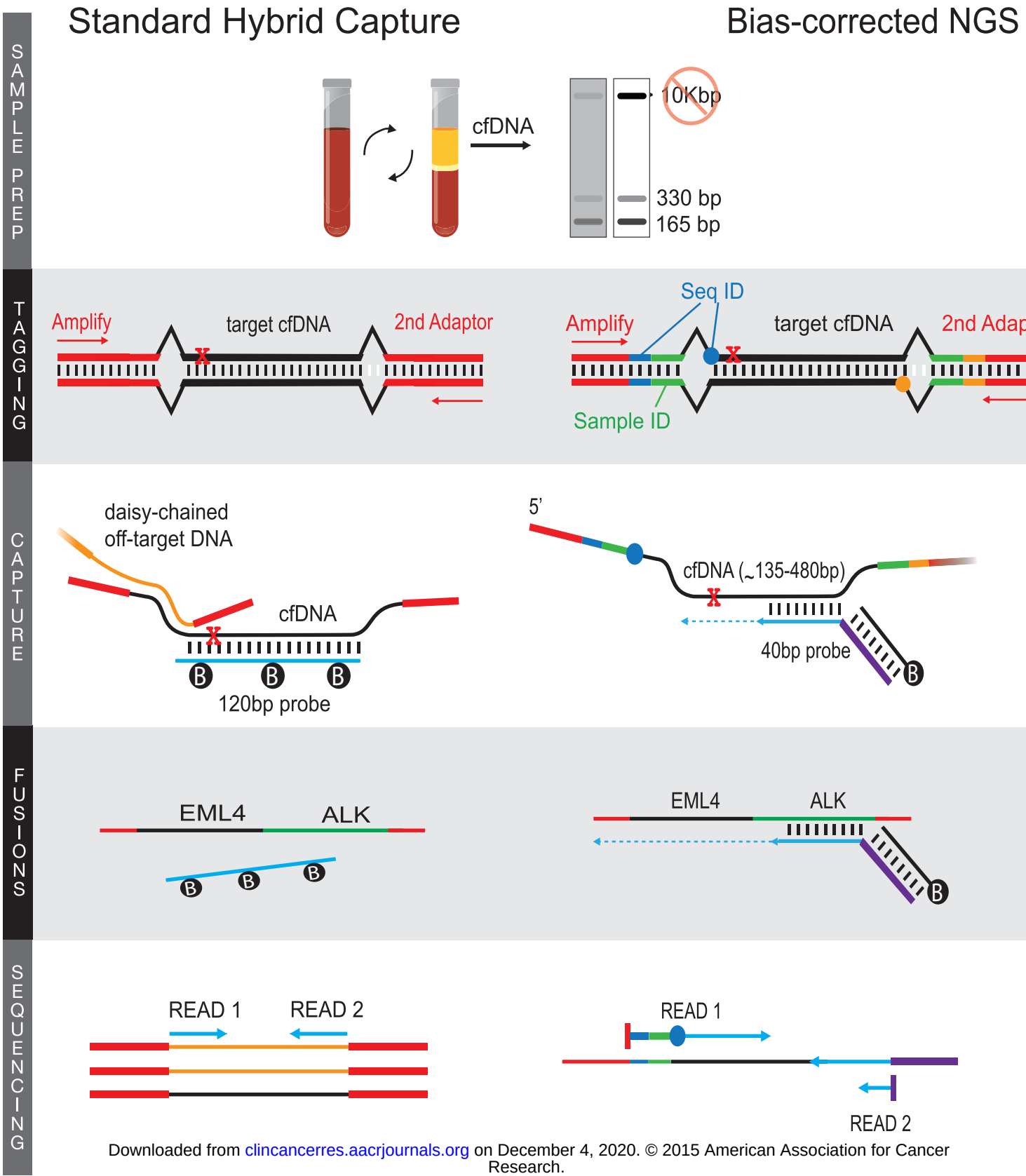
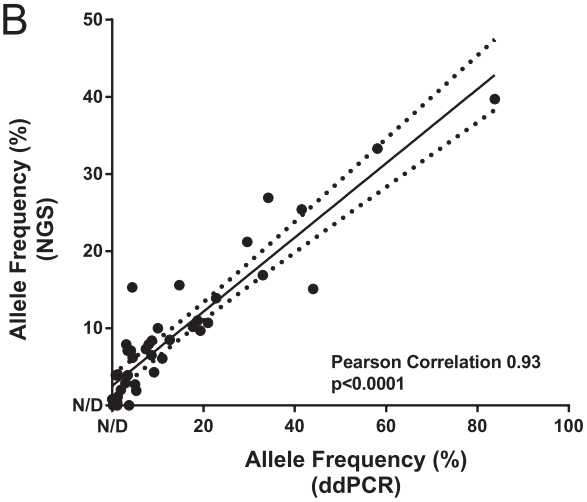


Figure 2

A

Sample	Tissue Genotype	ddPCR AF (%)	NGS AF (%)	GE in NGS library
105	*EGFR* del19	9.7	6.5	15733
116	*EGFR* del19	58	33	11852
179	*KRAS* G12C	4.1	7.1	8636
195	*EGFR* del19	19	11	5617
244	*EGFR* del19	18	10	4852
510	*EGFR* del19	0.1	0.4	4680
053	*EGFR* del19	2.9	2.0	3619
200	*KRAS* G12C	34	27	3206
091	*EGFR* del19	9.2	4.3	3021
081	*KRAS* G12C	5.1	3.9	2071
004	*EGFR* del19	11	6.1	1672
044	*EGFR* del19	21	11	1510
522	*EGFR* del19	30	14	1480
001	*KRAS* G12C	1.9	2.0	1256
120	*EGFR* del19	84	40	1202
011	*EGFR* del19	5.3	1.9	1087
017	*EGFR* del19	44	15	1035
232	*EGFR* L858R	3.4	7.1	1013
039	*EGFR* del19	19	17	1001
095	*EGFR* del19	33	9.7	746
048	*EGFR* L858R		0.8	605
061	*KRAS* G12C	0.2		463
045	*EGFR* del19			382
028	*EGFR* L858R	1.1		382
070	*KRAS* G12C	1.2	1.1	300
008	*KRAS* G12C	3.7		289
074	*EGFR* del19	3.1	7.9	100
094	*KRAS* G12V			89
109	*EGFR* del19			17

B

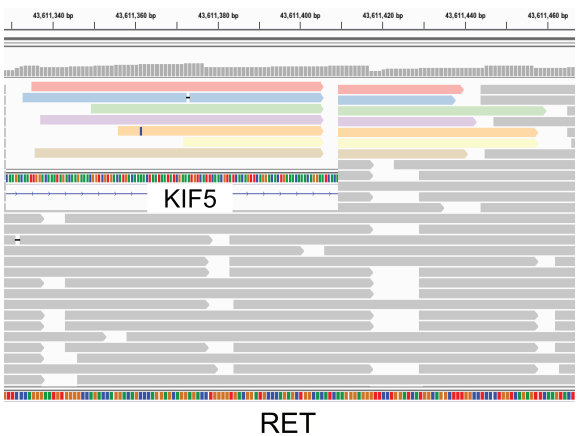


C

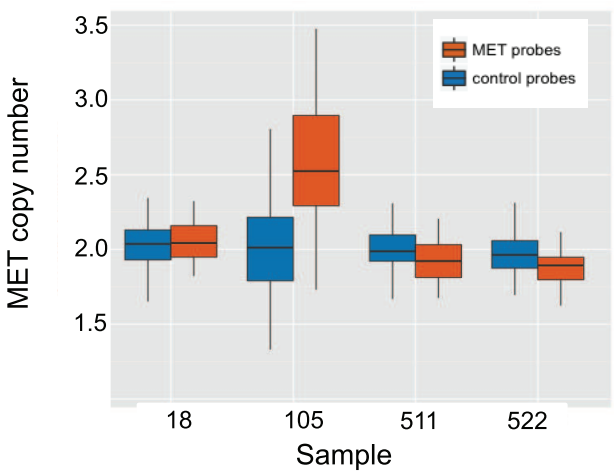
Sample	Tissue Genotype	NGS AF (%)	GE in NGS library
133	*KRAS* Q61H	5	11810
130	*EGFR* Ex20ins	40	7734
250	*EGFR* Ex20ins	13	6365
209	*ALK* fusion		5832
127	*EZR-ROS1*	0.5	4562
018	*EML4-ALK*	4.0	4429
015	*CD74-ROS1*	0.4	3990
081	*PIK3CA* E545K	3.9	2071
169	*RET* fusion		1719
036	*EGFR* G719A	6.0	1253
107	*KIF5B-RET*	21	859
022	*EGFR* G719A	2.1	858
145	*EGFR* Ex20ins	25	826
903	*Her2-neu 2311-2322 dup*	14	696
137	*KIF5B-RET*	4.0	475
115	*KRAS* Q61L		420
089	*KRAS* G13D		312
108	*BRAF* V600E		265
904	*Her2-neu 2332-2340 dup*	8.0	181
202	*EML4-ALK*	0.5	180

Figure 3

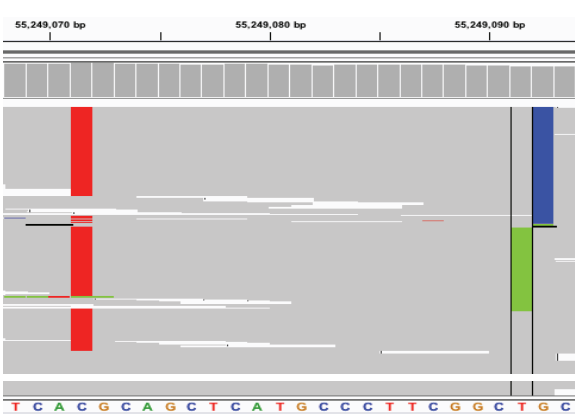
A



B



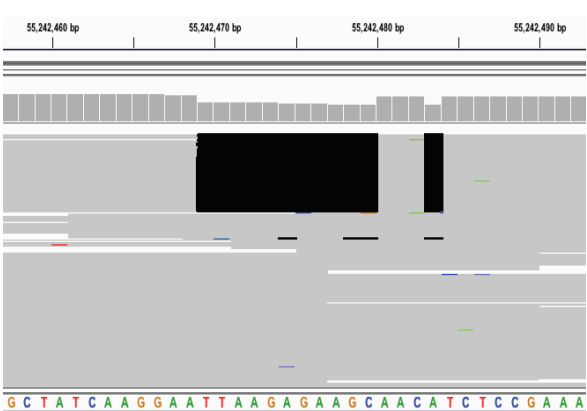
C



EGFR T790M

EGFR C797S

D



EGFR KELREATSPK>KENSPK

Author Manuscript Published OnlineFirst on October 12, 2015; DOI: 10.1158/1078-0432.CCR-15-1627-T
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Table 1: Detection of resistance mechanisms using plasma NGS compared to tissue genotyping of re-biopsy specimens in patients with acquired resistance to kinase inhibitors.

Sample	Baseline genotype	Therapy received	Tissue genotype at resistance	Plasma NGS at resistance	Allele frequency
11	*EGFR* exon 19 del	erlotinib	*EGFR* exon 19 del *EGFR* T790M	*EGFR* exon 19 del *EGFR* T790M	1.9% 0.5%
17	*EGFR* exon 19 del	erlotinib	*EGFR* exon 19 del *EGFR* T790M	*EGFR* exon 19 del *EGFR* T790M	15.1% 10.0%
39	*EGFR* exon 19 del	erlotinib	*EGFR* exon 19 del *EGFR* T790M	*EGFR* exon 19 del *EGFR* T790M	9.7% 2.7%
44	*EGFR* exon 19 del	erlotinib	*EGFR* exon 19 del *EGFR* T790M	*EGFR* exon 19 del *EGFR* T790M	10.7% 7.3%
48	*EGFR* L858R	afatinib	*EGFR* L858R *EGFR* T790M	*EGFR* L858R	0.8%
74	*EGFR* exon 19 del	erlotinib	*EGFR* exon 19 del *EGFR* T790M	*EGFR* exon 19 del	7.9%
91	*EGFR* exon 19 del	erlotinib	*EGFR* exon 19 del *EGFR* T790M	*EGFR* exon 19 del *EGFR* T790M	4.3% 3.9%
95	*EGFR* exon 19 del	erlotinib	*EGFR* exon 19 del *EGFR* T790M	*EGFR* exon 19 del *EGFR* T790M	16.9% 15.6%
22	*EGFR* G719A	erlotinib	*EGFR* G719A	*EGFR* G719A	2.1%
105	*EGFR* exon 19 del	afatinib	*EGFR* exon 19 del *MET* amp	*EGFR* exon 19 del *MET* amp	6.5% NC
522	*EGFR* exon 19 del *EGFR* T790M	AZD9291	*EGFR* exon del 19 *EGFR* T790M *EGFR* C797S	*EGFR* exon del 19 *EGFR* T790M *EGFR* C797S	13.9% 8.5% 7.8%
18	*ALK* rearrangement	crizotinib	(No tissue available)	*EML4-ALK* *ALK* G1128A *ALK* G1156Y	4.0% 2.0% 1.6%
127	*ROS1* rearrangement	crizotinib	*ROS1-EZR*	*ROS1-EZR*	0.5%
120	*EGFR* exon 19 del	erlotinib	*EGFR* exon 19 del *EGFR* T790M	*EGFR* exon 19 del *EGFR* T790M	39.7% 8.4%
232	*EGFR* L858R	erlotinib	*EGFR* L858R *EGFR* T790M	*EGFR* L858R *EGFR* T790M	7.1% 6.2%

AACR American Association for Cancer Research

Clinical Cancer Research

Bias-corrected targeted next-generation sequencing for rapid, multiplexed detection of actionable alterations in cell-free DNA from advanced lung cancer patients

Cloud P Paweletz, Adrian Sacher, Chris K Raymond, et al.

Clin Cancer Res Published OnlineFirst October 12, 2015.